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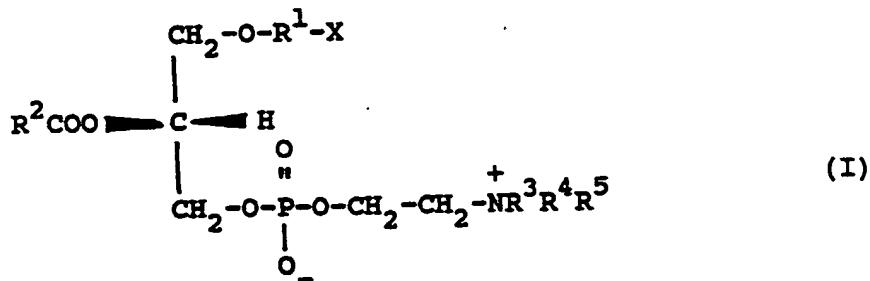
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(54) Title: ANTIGENIC ANALOGUES OF PLATELET ACTIVATING FACTOR (PAF)



(57) Abstract

Antigens for the production of antibodies to Platelet Activating Factor (PAF). The antigens are PAF analogues of formula (I), wherein X comprises a high molecular weight group, R¹ is a linking group and R² to R⁵ are selected from C₁ to C₆ alkyl. Other aspects of the invention include PAF-antibodies produced using said antigens, labelled PAF analogues, intermediates for the preparation of PAF analogues and methods and a kit for the immunoassay of PAF.

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ANTIGENIC ANALOGUES OF PLATELET ACTIVATING FACTOR (PAF)Technical Field

The present invention relates to novel antigens capable of producing antibodies to Platelet Activating Factor (PAF), novel PAF analogues labelled to enable quantitative assay, intermediates for the production of novel PAF antigens and methods for the preparation of said antigens, and methods of immunoassay of PAF in biological fluid using said labelled analogues and/or 10 labelled PAF-antibodies.

Background

Platelet Activating Factor (PAF),
15 1-O-alkyl-2-O-acetyl-sn-glycero-3-phosphocholine, represents a recently defined example of a class of biologically-active lipids active in the subnanomolar range and possessing a wide spectrum of pathophysiological effects. PAF promotes life threatening anaphylactic reactions in animals and is suspected of mediating a range of allergic and inflammatory reactions in man. For example, PAF may be important in conditions such as asthma, adult respiratory distress syndrome and shock reactions. However, despite 20 the increasing catalogue of conditions in which PAF maybe involved, greater insights into its role in health and disease are hampered because precise and specific methods

for its measurement are lacking. The capacity of PAF to aggregate platelets does not provide a suitable basis for strictly quantitative assay.

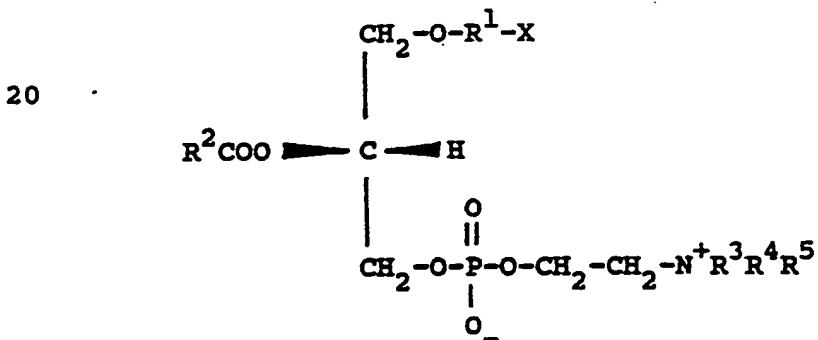
It would be desirable to develop an immunoassay for quantitative determination of PAF levels in blood serum. However, it has been found that PAF itself is insufficiently antigenic to produce the necessary PAF-antibodies needed for such an immunoassay.

Novel synthetic PAF analogues have now been found which are sufficiently antigenic to produce PAF-antibodies and a method suitable for the immunoassay of PAF levels in biological fluids has been developed.

The Invention

15

Accordingly the invention provides novel compounds of general formula (I) ...



25 wherein:

- (1) R^1 is a C_2 to C_{25} alkylene or alkenylene linking group substituted by radioactive iodine;
- X is hydrogen; or

(2) R^+ is a C_2 to C_{25} alkylene, alkenylene or alkynylene linking group optionally substituted by tritium or radioactive iodine;

X is selected from:

5 (a) the group consisting of formyl, di(C_1 to C_6 alkoxy)methyl, carboxy, isothiocyanato, $N-C_1$ to C_6 alkylamino, N,N -di(C_1 to C_6 alkyl)amino, hydroxy and mercapto; and

10 (b) the group -A-B wherein A is a linking group selected from the groups $-NR^6-$, $-COO-$, $-OCO-$, $-CONR^6-$, $-NR^6CO-$, $-NH-CS-NH-$ and $-S-S-$ wherein R^6 is selected from hydrogen and C_1 to C_6 alkyl; and

B is selected from:

15 (i) monofunctional and polyfunctional protein peptide, carbohydrate and lipid groups and derivatives thereof of molecular weight of at least 2000; and

20 (ii) a label; and

R^2 to R^5 are independently selected from C_1 to C_6 alkyl; and mixtures of the compound of formula (I) and its enantiomer.

25 In one embodiment the invention provides antigenic PAF analogues of general formula (I) wherein:

R^1 is a C_2 to C_{25} alkylene or alkenylene linking group;

X is the group -A-B wherein:

- A is a linking group selected from -NR⁶-, -COO-, -OCO-, -CONR⁶-, -NR⁶CO- and -S-S- wherein R⁶ is selected from hydrogen and C₁ to C₆ alkyl; and
- 5 B is selected from monofunctional and polyfunctional protein, peptide, carbohydrate and lipid groups and derivatives thereof of molecular weight of at least 2000 which are capable of eliciting an antigenic response; and
- 10 R² to R⁵ are independently selected from C₁ to C₆ alkyl.

In the antigenic PAF analogues of the invention of general formula (I):

- Preferred R¹ include straight chain C₄ to C₁₆ alkylene.
- 15 More preferred R¹ include straight chain C₄ to C₈ alkylene. For convenience R¹ is often chosen from pentylene and hexylene.
- Preferred A include -NR⁶-, -COO-, -OCO-, -CONR⁶- and R⁶CO- and preferred R⁶ include hydrogen and methyl. More
- 20 preferred A include -NR⁶- and -OCO-.
- Preferred B include monofunctional and polyfunctional protein, peptide, carbohydrate and lipid groups of molecular weight at least 5000 and capable of eliciting an antigenic response. More preferred B include
- 25 monofunctional and polyfunctional groups of molecular weight at least 10,000. Examples of suitable B include Bovine Serum Albumen (BSA), ovalbumen, Porcine Thyroglobulin (PTG), Bovine Thyroglobulin (BTG), keyhole

limpet haemocyanin, bacterial cell walls, synthetic polypeptides such as polylysine, poke weed mitagen (PWM), phytohaemagglutinin (PHA), muranyl dipeptidase and lipopolysaccharides.

5 -Preferred R² to R⁵ include C₁ to C₃ alkyl, and especially methyl.

In another embodiment the invention provides labelled PAF analogues of general formula (I) wherein:

- 10 (1) R¹ is a C₂ to C₂₅ alkylene or alkenylene linking group substituted by radioactive iodine;
X is hydrogen; or
(2) R¹ is a C₂ to C₂₅ alkylene, alkenylene or alkynylene linking group;
15 X is a group of formula -A-B wherein:
A is a linking group selected from -NR⁶-, -COO-
'
-OCO-, -CONR⁶-, -NR⁶CO-, -NH-CS-NH-and -S-S-
wherein R⁶ is selected from hydrogen and C₁ to C₆ alkyl;
20 B is a label; and
R² to R⁵ are independently selected from C₁ to C₆ alkyl.

In the labelled PAF analogues of the invention of general formula I wherein X is hydrogen:

- 25 -Preferred R¹ include straight chain C₄ to C₁₆ alkylene or alkenylene substituted by radioactive iodine.
-Preferred R² to R⁵ are methyl.

In the labelled PAF analogues of the invention of general formula I wherein X is a group of formula -A-B:

-Preferred R¹ include straight chain C₄ to C₁₆ alkylene, alkenylene or alkynylene. More preferred R¹ include

5 straight chain C₄ to C₈ alkylene.

-Preferred A include -NR⁶-, -COO-, -OCO-, -CONR⁶- and -NR⁶CO- and preferred R⁶ include hydrogen and methyl. More preferred A include -NR⁶- and -OCO-.

-In this specification, "label" is used to mean conventional labels used in immunoassay procedures including : the radioactive isotope labelled groups based on ¹²⁵I-histamine, ¹²⁵I-tyramine, ¹²⁵I-tyrosine methyl ester and ¹²⁵I-Bolton Hunter Reagent; enzymic labels; and photometric labels. Specific examples emzymic labels

10 include horseradish peroxidase, alkaline phosphatase, betagalactosidase and urease. Specific examples of photometric labels include fluorescent groups such as fluorescein and its derivatives, rhodamine and its derivatives, phycoerythrins, europium, "Texas Red",

15 luminescent labels such as luminol and its derivatives, acridinium esters and umbelliferins.

-Preferred R² to R⁵ are C₁ to C₃ alkyl, especially methyl.

25 In another embodiment the invention provides

compounds of general formula (I) which are useful as intermediates for the preparation of the antigenic PAF analogues of the invention wherein:

R¹ is a C₂ to C₂₅ alkylene, alkenylene or alkynylene
5 linking group; and

X is selected from the group consisting of formyl,
carboxy, di(C₁ to C₆ alkoxy)methyl, N-C₁ to C₆
alkylamino, N,N-di(C₁ to C₆ alkyl)amino, hydroxy and
mercapto.

10 In the intermediate compounds of the invention of
general formula I:

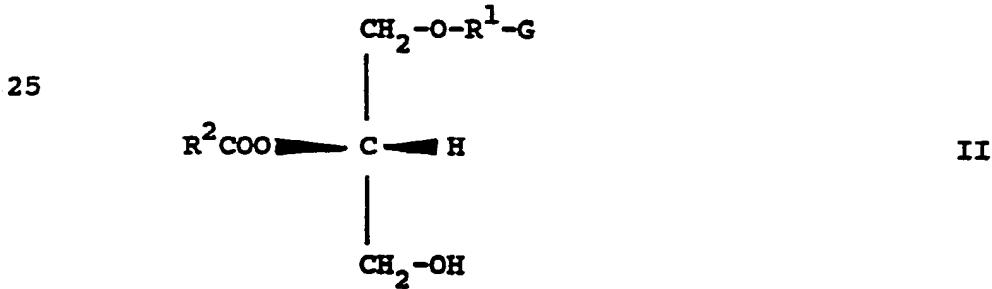
-Preferred R¹ include straight chain C₄ to C₁₆ alkylene,
alkenylene and alkynylene. More preferred R¹ include
straight chain C₄ to C₈ alkylene.

15 -Preferred X include formyl, carboxy, dimethoxymethyl and
hydroxy.

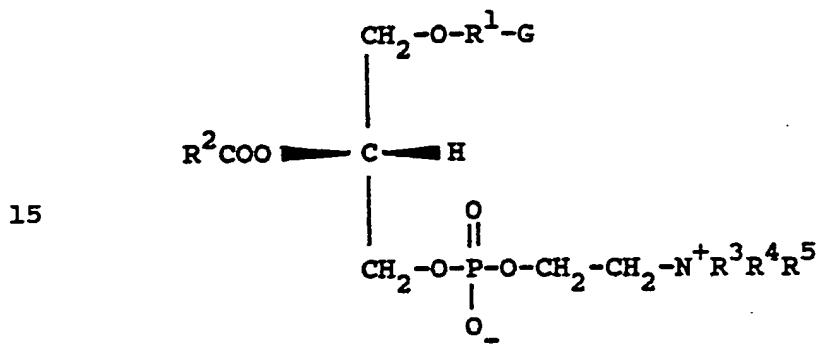
In another embodiment the invention provides
a process for the preparation of compounds of general
20 formula (I) which process comprises:

(a) reacting:

a compound of general formula (II)



wherein R¹ and R² are as hereinbefore defined and G
 is selected from di(C₁ to C₆ alkoxy)methyl and
 groups which may be reacted, using conventional
 methods, to give a group selected from formyl, di(C₁
 5 to C₆ alkoxy)methyl, carboxy, amino, N-C₁ to C₆
 alkylamino, N,N-di(C₁ to C₆ alkyl)amino, hydroxy and
 mercapto;
 a phosphorylation agent; and
 an N,N,N-tri(C₁ to C₆ alkyl)ethanolamine derivative
 10 to give a compound of general formula (III)



(b) reacting the product of (a) to convert group G as
 20 hereinbefore defined to a group selected from
 formyl, di(C₁ to C₆ alkoxy)methyl, carboxy, amino,
 N-C₁ to C₆ alkylamino, N,N-di(C₁ to C₆ alkyl)amino,
 hydroxy and mercapto and to introduce the desired
 group X.

25

In a specific example of the process for the
 preparation of compounds of general formula (I):

- (a) a compound of general formula (II), wherein G is dimethoxymethyl, R¹ is selected from C₄ to C₁₆ alkylene, alkenylene and alkynylene and R² is methyl, is reacted with phosphorus oxychloride and choline tosylate to give a compound of formula (III), wherein G is dimethoxymethyl, R¹ is selected from C₄ to C₁₆ alkylene, alkenylene and alkynylene, and R² to R⁵ are methyl; and
- 5 (b) the product of (a) is reacted with acid to give a compound of formula (I) wherein X is formyl and R¹ to R⁵ are as hereinbefore defined, which is reacted with a protein or synthetic peptide followed by reduction of the resulting imine to give a compound 10 of general formula (I) wherein R¹ to R⁵ are as hereinbefore defined and X is the group -A-B wherein A is the linking group -NR⁶- in which R⁶ is 15 hydrogen and B is a protein or synthetic peptide.

It will be recognized by those skilled in the art that in those antigenic PAF analogues of general formula I the group B may be monovalent or polyvalent such that a plurality of residues of general formula (I), typically between 1 and 500 and usually between 2 and 20, are attached to each group B. Therefore, in those 20 antigenic PAF analogues of general formula I in which X is the group -A-B, if the residue of formula (I) is represented by Z then the invention includes antigenic PAF analogues of formula (Z)_nB wherein n is an integer 25 from 1 to 500.

It will also be recognized by those skilled in the art that certain of the PAF analogues of general formula (I) may be non-covalently bonded to or adsorbed onto a solid support. Accordingly in another embodiment the invention provides supported PAF analogues comprising PAF analogues of general formula (I) wherein:

5 (1) R^1 is a C_2 to C_{25} alkylene or alkenylene linking group substituted by radioactive iodine; X is hydrogen; or

10 (2) R^1 is a C_2 to C_{25} alkylene, alkenylene or alkynylene linking group optionally substituted by tritium or radioactive iodine;

X is selected from:

15 (a) the group consisting of formyl, di(C_1 to C_6 alkoxy)methyl, carboxy, isothiocyanato, $N-C_1$ to C_6 alkylamino, N,N -di(C_1 to C_6 alkyl) amino, hydroxy and mercapto; and

20 (b) the group -A-B wherein A is a linking group selected from the groups $-NR^6-$, $-COO-$, $-OCO-$, $-CONR^6-$, $-NR^6CO-$, $-NH-CS-NH-$ and $-S-S-$ wherein R^6 is selected from hydrogen and C_1 to C_6 alkyl; and B is a label; and

25 R^2 to R^5 are independently selected from C_1 to C_6 alkyl; non-covalently bonded to or adsorbed onto a solid support material.

Examples of solid support materials for said supported PAF analogues include proteins, synthetic polypeptides (eg polylysine) carbohydrates and carbohydrate derivatives [e.g. nitrocellulose, agaroses

such as "Sephadex" (Trade Mark), and
lipopolysaccharides] and synthetic polymers such as, for
example, polysulphones, polyamides (e.g. polyacrylamide,
nylon 6, nylon 66, nylon 610) and polystyrene in the form
5 of particles, balls or formed articles such as test-
tubes, rods, tubes, fins, wells, beads, disks, slides,
plates and micro-titre plates.

Although PAF itself has been found to be
10 insufficiently antigenic to produce the PAF-antibodies
required to develop an immunoassay for PAF, surprisingly
it has been found that:

- (a) PAF adsorbed onto or non-covalently bound to a
monofunctional or polyfunctional protein, peptide,
15 carbohydrate, lipid or a derivative thereof of
molecular weight at least 2000 and capable of
eliciting an antigenic response; and
- (b) the antigenic PAF analogues of general formula (I);
stimulate the production of antibodies which are
20 antibodies to PAF. Accordingly in a further embodiment
the invention provides antibodies to PAF and methods for
their production. Such antibodies, hereinafter referred
to as PAF-antibodies or anti-PAF, may be prepared by
those techniques known in the art and conventionally
25 involve introducing an antigenic PAF analogue of general
formula (I) into an animal such as a rabbit, mouse,
donkey, sheep, etc. to produce antibodies to the antigen
and isolating and purifying the antibodies. The
PAF-antibodies of the invention may be labelled with any
30 of the conventional labels used in immunoassay

procedures. Such labels include, for example, radioactive labels, enzymic labels and photometric labels such as those hereinbefore described.

5 The PAF antibodies of the invention include both monoclonal antibodies and polyclonal antibodies and techniques known in the art may be utilized to prepare the required type of antibody. For example, monoclonal antibodies may be produced using the antigenic
10 PAF analogues of general formula (I) of the invention by the techniques taught by G. Kohler and C. Milstein, Nature, 256, 495-497 (1975).

The PAF analogues and PAF antibodies of the invention may be used to qualitatively and quantitatively analyse for the presence of PAF in biological fluids.
15 Accordingly in a further embodiment the invention provides methods for the immunoassay of PAF in biological fluids using the PAF analogues and/or PAF-antibodies of the present invention.

20 In one method PAF or PAF analogue is immobilised on a solid support and reacted with labelled or unlabelled PAF-antibodies in the presence of known amounts of competing free PAF to generate a graph showing percent inhibition versus PAF concentration. If, unlabelled PAF
25 antibody is used the antibody bound which binds to the first is detected by using a labelled second antibody (goat, donkey, sheep, etc.). Using this graph the amount of free PAF in biological fluids may be quantitatively measured.

30 In another method, unlabelled anti-PAF bound to a

solid support is reacted with a polyvalent antigenic PAF analogue of formula $(Z)_n B$ (e.g. PAF-polylysine). The resulting complex is then determined using labelled anti-PAF which binds to free PAF residues on the polyvalent 5 antigenic PAF analogue.

In another method, unlabelled anti-PAF bound directly, either covalently or non-covalently, to a solid phase such as magnetized particles, plastic tubes, micro-titre plates, "Sephadex" (Trade Mark) particles, 10 polyacrylamide particles, nylon or polystyrene balls, etc. is mixed in a competition assay with: (a) a known quantity of labelled PAF; and (b) known quantities of unlabelled PAF contained in standard solutions or PAF to be measured in an extract or biological fluid. The 15 concentration of unlabelled PAF in the sample is then determined from a standard curve, for example from a logit/log standard plot.

In another method, the procedure above is used except that the anti-PAF is linked to the solid phase by 20 a ligand such as an antibody, protein A, lectin or an enzyme, for example:

-solid phase/sheep (or some other species) anti-rabbit (or mouse etc.) immunoglobulin/rabbit (or mouse etc.) anti-PAF; and
25 -solid phase/protein A/rabbit (or mouse etc.) anti-PAF.

In another method, anti-PAF, labelled PAF and PAF to be measured are mixed and the free PAF and antibody-bound PAF are separated using dextran-coated charcoal or some other solid phase adsorbent such as hydroxyapatite etc.
30 The concentration of unlabelled PAF in the sample being

measured is then determined from a standard curve.

In another method, anti-PAF/PAF complexes are precipitated with a second antibody or with a protein precipitating reagent such as ammonium sulphate.

5 Again, concentrations of unlabelled PAF may be determined from a standard curve.

In a further embodiment the invention also provides a kit for the immunoassay of PAF in a biological fluid said kit comprising PAF-antibodies of the present
10 invention.

In practice, it has been found that the PAF present in biological fluids such as blood serum is rapidly degraded by the enzyme PAF-acetylhydrolase which is also normally present in blood serum. Therefore, it
15 is preferable to first deactivate the enzyme. Three methods have been published for the deactivation of the enzyme, namely use of 1N hydrochloric acid, use of diisopropylfluorophosphate, and use of phenylmethanesulphonyl fluoride, but these methods suffer
20 the disadvantages of use of drastic conditions and/or toxic substances.

It has now been found that the addition of a detergent to the biological fluid sufficiently deactivates the enzyme to enable PAF to be quantitatively determined.
25 Therefore, in a further embodiment the invention provides a method of immunoassay of PAF in biological fluid which comprises diluting the fluid with an aqueous detergent solution, prior to subjecting the diluted fluid to an immunoassay. Preferably the detergent is a non-ionic
30 detergent, such as those selected from the group

consisting of: polyalkylene glycols; alcohol, phenol and alkylphenol alkoxylates; castor oil alkoxylates; the partial esters derived from long chain fatty acids and hexitol anhydrides and their alkoxylates; long chain 5 alcohol polyglycol ether acetals; alcohol sugar acetals; and the lecithins. Detergents such as "Tween"20, "Nonidet" P40 and "Triton" X100 (Trade Marks) have been found particularly useful.

10 Industrial Applicability

It will be evident to those skilled in the art that the products and methods of the invention find particular use in the medical and veterinary fields for 15 the analysis of PAF.

Preferred Embodiments

Embodiments of the present invention will now be described by way of example only.

5

Example 1Preparation of 2-O-Acetyl-1-O-(6',6'-dimethoxyhexyl)-sn-glyceryl-3-phosphorylcholine

10

1. 1,1-Dimethoxycyclohexane.

A mixture of cyclohexanone (52 ml, 0.5 mol), trimethyl-orthoformate (66 ml, 0.6 mol), methanol (51 ml, 1.26 mol) 15 and concentrated H₂SO₄ (1 drop) was refluxed for 18 hours. A solution of sodium methoxide in methanol was added until the mixture was neutral, and the mixture was fractionally distilled. 1,1-Dimethoxycyclohexane was obtained from the fraction b.p. 162-164°C (50.6 g, 70%).

20

2. 1-Methoxycyclohexene.

1,1-Dimethoxycyclohexane (25 g, 0.174 mol) was heated with p-toluenesulfonic acid (35 mg) at 140°C for 3 hrs. 25 Methanol was distilled off during the reaction. The residue was fractionally distilled, yielding 1-methoxycyclohexene (15.2 g, 80%) b.p. 144-146°C.

3. Methyl 6,6-dimethoxyhexanoate.

A solution of 1-methoxycyclohexene (4.5 g, 0.04 mol) in methanol (140 ml) was ozonolysed at 0°C until
5 the uptake of ozone ceased. The solution was degassed and a suspension of reduced Pd/CaCO₃ (1.0 g) catalyst in methanol (30 ml) was added. The mixture was filtered through celite, and the filtrate was evaporated. Trimethylorthoformate (7
10 ml, 0.06 mol), methanol (5 ml, 0.12 mol) and conc.H₂SO₄ (1 drop) were added to the residue. After 17 hours, the mixture was neutralized with sodium methoxide solution and then fractionally distilled. Methyl
15 6,6-dimethoxyhexanoate was collected as the fraction b.p. 80-90°C/1.0 mm (4.1 g, 54%).

4. 6,6-Dimethoxyhexan-1-ol

To a stirred mixture of lithium aluminium hydride
20 (3.8 g, 0.1 mol) in ether (80 ml) under nitrogen, was added methyl 6,6-dimethoxyhexanoate (15.0 g, 0.079 mol) in ether (50 ml) at a rate to maintain reflux (ca.1.5 hr). The mixture was further refluxed for 1.5 hrs., and then cooled to 0°C. Sodium
25 hydroxide solution (13 ml, 7 M) was added dropwise while cooling in ice. After stirring for 1 hour, the mixture was filtered through a layer of magnesium sulfate. The residue was washed with ether, and the combined filtrates were evaporated. The residue was

subjected to "suction" chromatography.

6,6-Dimethoxyhexan-1-ol was eluted with 25% ethyl acetate in light petroleum (10.3 g, 80%).

5 5. 2-O-Acetyl-3-O-benzyl-1-O-(6',6'-dimethoxyhexyl)
-sn-glycerol

Sodium hydride dispersion (0.377 g, 12.6 mmol, 80% in oil) was washed with dry ether under nitrogen.

The residue was resuspended in dry DMF (30 ml), and 6,6-dimethoxyhexan-1-ol (1.62 g, 10 mmol) was added.

The mixture was heated at 80°C for 1.25 hr., during which time the sodium hydride reacted. (R)-1-(Benzylxy)-2,3-epoxypropane (1.64 g, 10 mmol) was added and heating was continued for 2 hr. Upon

cooling, water (100 ml) was added and the mixture was extracted with ether (100 ml, 2 x 40 ml). The combined extracts were washed with water (2 x 80 ml) and brine (100 ml), dried ($MgSO_4$) and evaporated.

The residual oil (2.8 g) was dissolved in chloroform (36 ml), and cooled to 0°C. Pyridine (3.5 ml, 43 mmol) and freshly distilled acetyl chloride (0.94 ml, 13.2 mol) were added. The mixture was stirred for 0.5 hr. at 25°C, then 2 hr. at room temperature

(RT). Ice water (100 ml) was added and the layers separated. The aqueous layer was extracted with chloroform (2 x 40 ml), and the combined organic phases were washed with water (100 ml) and brine (100 ml), dried ($MgSO_4$) and evaporated. The residue was subjected to chromatography and the product was eluted with petroleum ether-ethyl acetate (9:1). Evaporation of this fraction yielded the product as a colorless oil (1.82 g, 50%) b.p. $170^\circ C/0.2$ mmHg (C₂₀H₃₂O₆ requires C, 65.19; H, 8.75%, Found: C, 65.06%; H, 8.66%), $[\alpha]_D + 1.98^\circ$ (c 5.06, benzene). ¹H N.M.R. δ : 7.36, m, 5, ArH; 5.17, q, 1, J 5.0 Hz, H2; 4.50, d, 2, J 2.5 Hz, benzyl; 4.36, t, 1, J 5.7 Hz, -CH(OMe)₂; 3.62, d, 2, J 5.0 Hz, H3; 3.58, d, 2, J 5.2 Hz, OCH₂-; 3.48-3.39, m, 2, H1; 3.31, s, 6, OCH₃; 2.12, s, 3, COCH₃; 1.72-1.26, m, 8, -CH₂-.

Mass spectrum: m/e 337, 305, 287, 245, 229, 215, 207, 146, 117, 113, 111, 91, 81, 75, 72.

20 6. 2-O-Acetyl-1-O-(6',6'-dimethoxyhexyl)-sn-glycerol

2-O-Acetyl-3-O-benzyl-1-O-(6',6'-dimethoxyhexyl)-sn-glycerol (369 mg, 1.0 mmol) was hydrogenated in THF (10 ml) over Palladium/carbon (14 mg, 10%) until the uptake of hydrogen ceased (approx. 2.5 hr.). The solution was filtered through celite, and the filtrate was evaporated to yield a colourless oil (278 mg, 100%) which was used immediately. ¹H N.M.R. δ : 5.04, q, 1, J 5.0 Hz, H2; 4.40, t, 1, J 5.7 Hz, -CH(OMe)₂; 3.84, d, 2, J 5.0 Hz, H3; 3.65, d, 2, J 5.2 Hz, OCH₂-; 3.56-3.44, m, 2, H1; 3.35, s, 6, OCH₃;

2.5, s(b), 1, OH; 2.14, s, 3, COCH₃; 1.7-1.3, m, 8, -CH₂-.

7. 2-O-Acetyl-1-O-(6',6'-dimethoxyhexyl)-sn-glyceryl
3-phosphorylcholine

To a stirred, cold (0°C) solution of distilled triethylamine (0.35 ml, 2.5 mmol) in dichloromethane (4 ml) under nitrogen, was added distilled phosphorous oxychloride (0.11 ml, 1.2 mmol) and then 2-O-acetyl-1-O-(6',6'-dimethoxyhexyl)-sn-glycerol (278 mg, 1.0 mmol) in dichloromethane (5 ml). The solution was stirred for 1 hr. at RT, and choline tosylate (465 mg, 1.7 mmol) in pyridine (10 ml) was added. Stirring was continued for 17 hrs. at RT. Sodium bicarbonate (0.4 g) and water (1 ml) were added and the mixture was evaporated at 30°C. The residue was extracted several times with chloroform (total 40 ml) and filtered. The filtrate was evaporated to yield a semi-solid residue (1.3 g).

An anion exchange column was prepared from DE-32 cellulose (5.5 g) in acetic acid, and washed successively with methanol, methanol/chloroform (1:1) and chloroform. The mixture (1.3 g) was applied to the column in a small volume of chloroform, and was then eluted with chloroform (100 ml), then methanol in chloroform (100 ml each of 1.5%, 3%, 4.5%, 6% v/v). The product was contained in the fractions 3-6% methanol in chloroform, as determined by t.l.c (CHCl₃/MeOH/H₂O

60:35:5). Evaporation of these combined fractions yielded a pale yellow semi-crystalline material (0.21 g), which was contaminated with a tosylate salt (approx. 30%). ^1H N.M.R. δ : 5.13, m, 1, H2; 5 4.37, t, 1, J 5.7 Hz, -CH(OMe)₂; 4.3-3.2, m, all other protons on C α to O or N; 2.06, s, 3, COCH₃; 1.7-1.3, m, 8, -CH₂- . ^{13}C N.M.R. δ 170.49, s, C=O; 10 104.25, s, -CH(OMe)₂; 71.90, d, J 8.0 Hz, C2; 71.17, s, -CH₂O(or N); 69.00, s, -CH₂O(or N); 65.76, s, -CH₂O (or N); 63.76, d, J 5.1 Hz; -CH₂OP; 59.03, d, J 4.4 Hz, -CH₂OP; 53.78, s, -N⁺(CH₃)₃; 53.38, s, OCH₃; 32.24, s, -CH₂-; 21.00, s, COCH₃.

Example 2

15

Preparation of 2-O-Acetyl-1-O-(6'-oxohexyl)-sn-glyceryl-3-phosphorylcholine

Crude 2-O-acetyl-1-O-(6',6'-dimethoxyhexyl)-sn-glyceryl-3 20 phosphorylcholine (130 mg) was suspended in ethyl acetate (9 ml) and aqueous trifluoroacetic acid (TFA) (170 μ l, 90%) was added. The mixture was allowed to stand at RT for 1.5 hr. and 4°C for 17 hrs., until the deprotection was complete by t.l.c. Toluene (9 ml) was added 25 and the mixture evaporated. The residue was repeatedly evaporated from ethyl acetate/toluene (1:1) (10 ml) and alternatively from toluene (10 ml). The mixture was

chromatographed on silica gel (70-230 mesh) and the product was eluted with CHCl₃/MeOH/H₂O (40:60:10). Evaporation of the appropriate fractions yielded a colorless oil (50 mg). ¹H N.M.R. δ : 9.78, t, 1, J 2.0 Hz, 5 CH=O; 5.1, m, 1, H2; 4.4-3.2, m, all other protons on C αC to O or N; 2.46, dt, 2, J 2.0 & 7.0 Hz, -CH₂-CHO; 2.08, s, 3, COCH₃; 1.7-1.3, m, 6, -CH₂-.. ¹³C N.M.R. δ : 176.05, s, -CHO; 170.79, s, -OCOCH₃; 72.07, s, C2; 71.21, s, -CH₂O (or N); 69.27, s, -CH₂O (or N); 66.12, s, 10 -CH₂O (or N); 64.11, s, -CH₂OP; 59.38, s, -CH₂OP; 54.20, s, -N⁺(CH₃)₃; 43.78, s, -CH₂CHO; 29.28, s, -CH₂-; 25.64, s, -CH₂-; 21.80, s, -CH₂-; 21.26, s, COCH₃.

15 Example 3Coupling 2-O-acetyl-1-O-(6'-oxohexyl)-sn-glyceryl-3-phosphorylcholine to methylated BSA (PAF-BSA)

20 Methylated bovine serum albumin (250 mg) was dissolved in methanol (90 ml) and 2-O-acetyl-1-O-(6'-oxohexyl)-sn-glyceryl-3-phosphorylcholine (25 mg) in methanol (5 ml) was added. The solution was left at RT for 0.5 hr., and then sodium cyanoborohydride (100 mg) was added. The 25 pH of the solution was adjusted to 5 with 1M HCl. After standing for 16 hr. at RT, the mixture was evaporated. The residue was dispersed in water (90 ml) and dialysed against distilled water (20 l). The dialysate was

freeze-dried to yield a fluffy white material (238 mg). This material was assayed for phosphorous content, which was found to be 100 nanomoles per mg.

5 Example 4

Coupling 2-O-acetyl-1-O-(6'-oxohexyl)-sn-glyceryl-3-phosphoryl-choline to polylysine (PAF-PL)

10 2-O-Acetyl-1-O-(6'-oxohexyl)-sn-glyceryl-3-phosphoryl-choline was coupled to the polyvalent synthetic polypeptide polylysine following essentially the same procedure as that described in Example 3.

15 Example 5

Inactivation of PAF-Acetylhydrolase

20 The following experiments demonstrate that PAF-acetylhydrolase can be deactivated by the addition of detergents.

Materials

25 PAF (from bovine heart lecithin) and "Tween" 20 (polyoxyethylene sorbitan monolaurate) were from Sigma (St. Louis, Mo., USA). Human sera albumin (HSA) was from Commonwealth Serum Laboratories (Melbourne, Australia).

Serum

Blood was collected from normal human donors by venipuncture, allowed to clot and the serum collected.

- 5 Serum was stored at -20°C until used. Similarly, rabbit serum was obtained from the ear veins of normal rabbits.

Platelet-Rich Plasma

10

Whole blood was collected from normal human donors, who had taken no medication for at least 10 days before venipuncture, and mixed with 0.1M trisodium citrate (0.1 vol). Platelet-rich plasma was produced by centrifugation (10 min, 600 r.p.m.) and was used within 1 hour.

15

Dilution of Sera

20

Sera were diluted 1 in 100 in either PBS or 0.1% "Tween" in PBS (v/v). Diluted acid-treated sera were prepared by mixing sera (1 vol.) with 0.1M citrate buffer pH 3.0 (2 vol.), and then 15 minutes later with PBS (98 vol.).

25

Determination of PAF-acetylhydrolase activity

30

Diluted serum ($50\mu\text{l}$) was incubated with 3.7×10^{-6} M PAF (in 2.5% HSA) ($50\mu\text{l}$) for 27 hours at 25°C. The solution ($50\mu\text{l}$) was then tested for platelet aggregation activity at 37°C in a Payton dual

channel aggregometer using human platelet-rich plasma (500 μ l).

5 RESULTS AND DISCUSSION

Two human sera and two rabbit sera, each with added PAF, were diluted by the three methods (PBS, "Tween" and acid-treated) and were then tested for acetylhydrolase activity. The results were in the form of light-transmission tracings from the aggregometer. After 27 hours incubation, PAF was destroyed in all sera diluted with PBS whereas the sera diluted in 0.1% "Tween" showed no inactivation of PAF. The "Tween"-diluted sera were tested for platelet aggregating activity, but no aggregation was observed. As a control for the above experiment, PAF was incubated with PBS or 0.1% Tween in PBS. In these experiments platelet aggregation activity was retained.

Disparity between human and rabbit sera was found when the sera were treated with acid. Whereas, rabbit sera no longer destroyed PAF, acid-treated human serum still had acetylhydrolase activity. Human and rabbit sera appear to have the same buffering capacity, so the disparity probably arises from varying acid-sensitivities of the two acetylhydrolases.

These results show that "Tween" 20 inactivates PAF-acetylhydrolase. Dilution in "Tween" is thus a simple and

mild method of inactivating PAF-acetylhydrolase and this finding will be of great importance in immunoassay procedures used to measure PAF in biological fluids.

5

Example 6

Preparation of PAF-antibodies

10

2-O-acetyl-1-O-(6'-oxohexyl)-sn-glyceryl-3-phosphoryl-choline coupled to methylated bovine serum albumin prepared as described in Example 3 (PAF-BSA) was used as an antigen in rabbits and the immunoglobulin fraction was 15 isolated from the rabbit anti-PAF serum produced by affinity chromatography on "Sepharose"/protein A.

The presence of PAF-antibodies in the isolated immunoglobulin fraction was determined by a direct binding 20 assay showing binding to tritium labelled PAF (^3H -PAF) as described below.

A sample of the immunoglobulin fraction (Ig) was mixed in an assay tube with a mixture (3-5mg) of "Sepharose" 25 (solid support) and protein A (a ligand to link the antibody to solid support) and ^3H -PAF in a total volume of 50 to 100 μl and incubated at room temperature overnight. The resulting mixture was centrifuged, washed twice with phosphate buffered saline containing 0.1% 30 "Tween" 20, centrifuged and the sediment transferred in

water (200 μ l) to the liquid scintillant "Aquasol" (3ml)
and counted in a liquid scintillation counter.

The results, tabulated below, indicate significant uptake
5 of ^{3}H -PAF by the immunoglobulin isolated from rabbits
treated with the PAF-BSA antigen in comparison to
"normal" immunoglobulin isolated from control rabbits.

Rabbit	Ig	^{3}H -PAF	Assay Count	^{3}H -PAF
No	(μ g)	(cpm)	(cpm)	Uptake (%)
1	20	28,123	5,046	17.9
1	10	28,123	5,124	18.2
1	5	28,123	3,967	14.1
2	20	28,123	4,449	15.8
2	10	28,123	3,001	10.7
2	5	28,123	2,189	7.8
Control	20	28,123	326	1.2
Control	10	28,123	492	1.7
Control	5	28,123	281	1.0
None	0	28,123	140	0.5

*"Sephadex", "Tween" and "Aquasol" are Trade Marks.

Example 7

The following experiments demonstrate the use of
5 PAF-antibodies of the present invention in a competition
or inhibition assay with a known quantity of labelled PAF
and known quantities of unlabelled PAF or PAF analogues
of the invention which can be used to establish standard
plots from which the quantity of PAF in sample can be
10 determined. They also demonstrate the binding of the
PAF-antibodies of the invention to PAF and the PAF
analogues of the invention (e.g. PAF-PL of Example 4) in
comparison to lyso-PAF, lecithin and lyso-lecithin.

15 A standard quantity of immunoglobulin containing
PAF-antibodies (Ig) prepared as described in Example 6
was mixed in an assay tube with a mixture (3-5mg) of
"Sepharose" and protein A, ^3H -PAF (22,676 cpm), and a
sample of a "test" substance for competitive binding to
20 PAF-antibodies in a total volume of 100 to 200 μl and the
mixture was incubated at room temperature overnight. The
resulting mixture was centrifuged, washed twice with
phosphate buffered saline containing 0.1% "Tween" 20,
centrifuged and the sediment transferred in water (200 μl)
25 to the liquid scintillant "Aquasol" (3ml) and counted in
a liquid scintillation counter.

The results, tabulated below, indicate:

- (i) PAF-antibodies of the present invention may be
5 used in a competition assay with known amounts
of radiolabelled PAF and PAF to develop a
standard plot for the quantitative
determination of PAF by competition assay; and
- 10 (ii) the specific binding of the PAF-antibodies of
the invention to PAF and the PAF-analogues of
the invention (e.g. PAF-PL)

Name	TEST SUBSTANCE ng	ASSAY COUNT cpm	ASSAY/CONTROL
			%*
PAF	5,000	228	4.1
PAF	500	598	18.6
PAF	50	1,602	57.8
PAF	5	2,316	85.7
PAF	0.5	2,561	95.2
PAF-PL	27,000	435	12.2
PAF-PL	2,700	662	21.1
PAF-PL	270	429	11.9
PAF-PL	27	1,329	47.1
PAF-PL	2.7	2,338	86.5

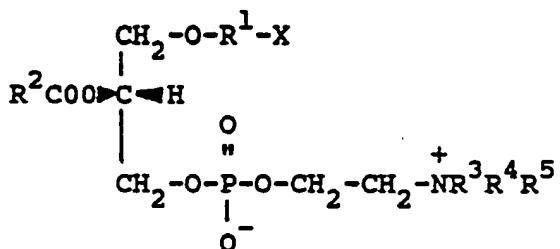
lyso-PAF	5,800	2,746	-
lyso-PAF	580	2,744	-
lecithin	5,000	2,994	-
lecithin	500	2,545	99.6
lyso-lecithin	5,000	2,658	99.0
lyso-lecithin	500	2,668	99.4
Control	0	2,683	100.0
No Ig	0	123	-

$$\frac{\text{Assay Count} - \text{Assay Count No Ig}}{\text{Assay Count Control} - \text{Assay Count No Ig}} \times 100$$

$$\text{i.e. } \frac{(\text{Assay Count} - 123)}{2560} \times 100$$

CLAIMS:

1. Compounds of general formula (I):



wherein:

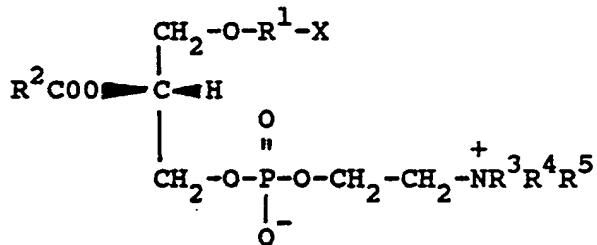
- (1) R^1 is a C_2 to C_{25} alkylene or alkenylene linking group substituted by radioactive iodine;
 X is hydrogen; or
- (2) R^1 is a C_2 to C_{25} alkylene, alkenylene or alkynylene linking group optionally substituted by tritium or radioactive iodine;
 X is selected from:
(a) the group consisting of formyl, di(C_1 to C_6 alkoxy)methyl, carboxy, isothiocyanato, N-C_1 to C_6 alkylamino, N,N-di(C_1 to C_6 alkyl)amino, hydroxy and mercapto; and
(b) the group -A-B wherein A is a linking group selected from the groups $-\text{NR}^6-$, $-\text{COO}-$, $-\text{OCO}-$, $-\text{CONR}^6-$, $-\text{NR}^6\text{CO}-$, $-\text{NH-CS-NH-}$ and $-\text{S-S-}$ wherein R^6 is selected from hydrogen and C_1 to C_6 alkyl; and B is selected from:
(i) monofunctional and polyfunctional protein, peptide, carbohydrate and lipid groups and derivatives thereof of

molecular weight of at least 2000; and

(ii) a label; and

R^2 to R^5 are independently selected from C_1 to C_6 alkyl; and mixtures of the compound of formula I and its enantiomer.

2. Antigenic PAF analogues of general formula (I)



wherein:

R^1 is a C_2 to C_{25} alkylene or alkynylene linking group;

X is the group -A-B wherein:

A is a linking group selected from $-\text{NR}^6-$, $-\text{COO}-$, $-\text{OCO}-$, $-\text{CONR}^6$, $-\text{NR}^6\text{CO}-$ and $-\text{S-S}$ wherein R^6 is selected from hydrogen and C_1 to C_6 alkyl; and

B is selected from monofunctional and polyfunctional protein, peptide, carbohydrate and lipid groups and derivatives thereof of molecular weight of at least 2000 which are capable of eliciting an antigenic response; and

R^2 to R^5 are independently selected from C_1 to C_6 alkyl.

3. Antigenic PAF analogues according to claim 2

wherein:

R^1 is selected from straight chain C_4 to C_{16} alkylene;

X is a group -A-B wherein:

A is selected from $-NR^6-$, $-COO-$, $-OCO-$, $-CONR^6-$ and $-NR^6CO-$ wherein R^6 is hydrogen or methyl; and

B is selected from monofunctional and polyfunctional protein, peptide, carbohydrate and lipid groups and derivatives thereof of molecular weight at least 5000 which are capable of eliciting an antigenic response; and R^2 to R^5 are independently selected from C_1 to C_3 alkyl.

4. Antigenic PAF analogues according to claim 2 or claim 3 wherein:

R^1 selected from straight chain C_4 to C_8 alkylene; X is a group $-A-B$ wherein:

A is selected from $-NH-$ and $-COO-$; and B is selected from monofunctional and polyfunctional protein and peptide groups of molecular weight at least 10,000 which are capable of eliciting an antigenic response; and R^2 to R^5 are each methyl.

5. Antigenic PAF analogues according to any one of claims 2 to 4 inclusive wherein:

R^1 is hexylene;

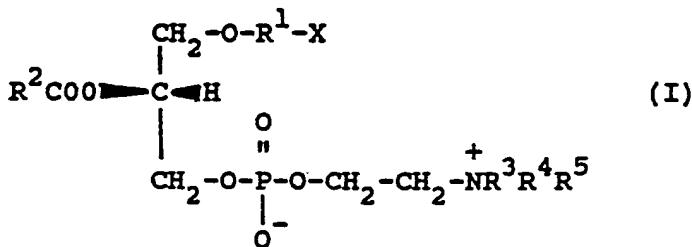
X is a group $-A-B$ wherein:

A is $-NH-$; and

B is selected from a protein residue derived from bovine serum albumen and a peptide residue derived from polylysine; and

R^2 to R^5 are methyl.

6. Labelled PAF analogues of general formula (I)



wherein

- (1) R^1 is a C_2 to C_{25} alkylene or alkenylene linking group substituted by radioactive iodine; X is hydrogen; or
 - (2) R^1 is a C_2 to C_{25} alkylene, alkenylene, or alkynylene linking group; X is a group of formula -A-B wherein:
 - A is a linking group selected from $-\text{NR}^6-$, $-\text{COO}-$, $-\text{OCO}-$, $-\text{OCO}-$, $-\text{CONR}^6$, $-\text{NR}^6\text{CO}-$, $-\text{NH-CS-NH-}$ and $-\text{S-S-}$ wherein R^6 is selected from hydrogen and C_1 to C_6 alkyl;
 - B is a label; and
- R^2 to R^5 are independently selected from C_1 to C_6 alkyl.

7. Labelled PAF analogues according to claim 6 wherein:

R^1 is selected from straight chain C_4 to C_{16} alkylene;

X is a group of formula -A-B wherein:

A is selected from $-\text{NR}^6-$, $-\text{COO}-$, $-\text{OCO}-$, $-\text{CONR}^6-$ and $-\text{NR}^6\text{CO}-$ wherein R^6 is hydrogen or methyl; and

B is labelled group selected from:

radiolabelled groups based on ^{125}I -histamine,

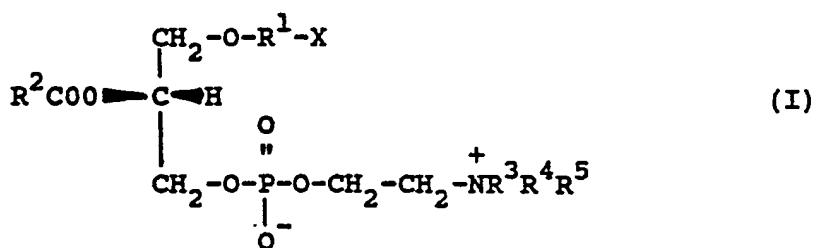
^{125}I -tyramine, ^{125}I -tyrosine methyl ester

and ^{125}I -Bolton Hunter Reagent; enzymic labels; and

photometric labels; and

R^2 to R^5 are independently selected from C_1 to C_3 alkyl.

8. Compounds of general formula (I) which are intermediates for the preparation of PAF analogues



wherein:

R^1 is a C_2 to C_{25} alkylene, alkenylene or alkynylene linking group; and

X is selected from the group consisting of formyl, carboxy, di(C_1 to C_6 alkoxy)methyl, N- C_1 to C_6 alkyl)amino, hydroxy and mercapto.

9. Compounds according to claim 8 wherein:

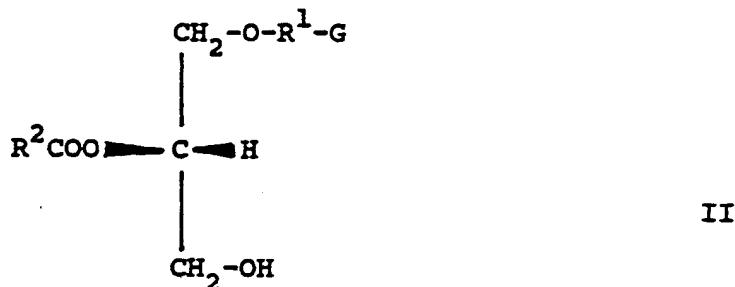
R^1 is selected from straight chain C_4 to C_{16} ; and

X is selected from formyl, carboxy, dimethoxymethyl and hydroxy.

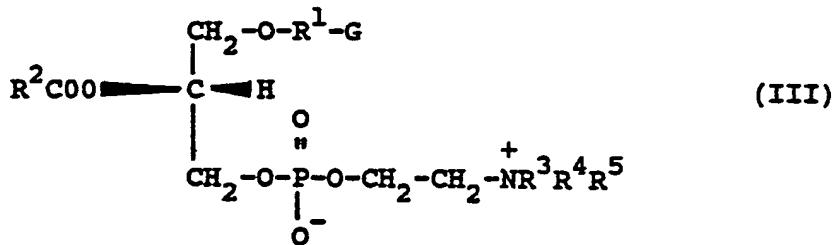
10. A process for the preparation of compounds of general formula (I) which process comprises:

(a) reacting:

a compound of general formula (II)



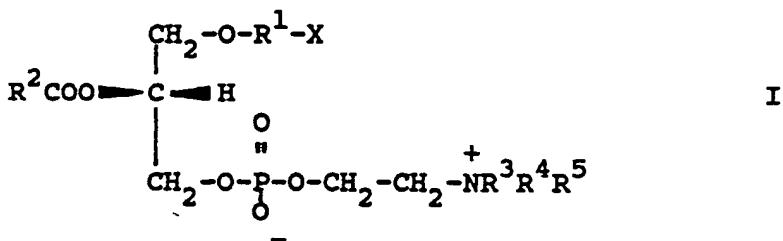
wherein R^1 and R^2 herein before defined and G is selected from di(C_1 to C_6 alkoxy)-methyl and groups which may be reacted, to give a group selected from formyl, di(C_1 to C_6 alkoxy)methyl, carboxy, amino, $N-C_1$ to C_6 alkylamino, N,N -di(C_1 to C_6 alkyl)amino, hydroxy and mercapto; a phosphorylation agent; and an N,N,N -tri(C_1 to C_6 alkyl) ethanolamine derivative to give a compound of general formula (III)



(b) reacting the product of (a) to convert group G as hereinbefore defined to a group selected from formyl, di(C₁ to C₆ alkoxy)methyl, carboxy, amino, N-C₁ to C₆ alkylamine, N,N-di(C₁ to C₆ alkyl)amino, hydroxy and mercapto and to introduce the desired group X.

II. Supported PAF analogues comprising:

PAF analogues of general formula (I)



wherein:

- (1) R¹ is a C₂ to C₂₅ alkylene or alkenylene linking group substituted by radioactive iodine;
X is hydrogen; or.
- (2) R¹ is a C₂ to C₂₅ alkylene, alkenylene or alkynylene linking group optionally substituted by tritium or radioactive iodine;
X is selected from:
 - (a) the group consisting of formyl, di(C₁ to C₆ alkoxy)methyl, carboxy isothiocyanato, N-C₁ to C₆ alkylamino, N,N-di(C₁ to C₆ alkyl)amino, hydroxy and mercapto; and

(b) the group - A-B wherein A is a linking group selected from the groups -NR⁶- , -COO-, -OCO-, =CONR⁶, -NR⁶CO-, -NH-CS-NH- and -S-S- wherein R⁶ is selected from hydrogen and C₁ to C₆ alkyl; and

B is a label; and

R² to R⁵ are independently selected from C₁ to C₆ alkyl; and a solid support material upon which said PAF analogues are covalently bound.

12. PAF antibodies prepared using as antigen:
 - (a) PAF adsorbed onto or non-covalently bound to a monofunctional or polyfunctional protein, peptide, carbohydrate, lipid or a derivative thereof of molecular weight at least 2000 and capable of eliciting an antigenic response; or
 - (b) the antigenic PAF analogues of general formula (I) as defined according to any one of claims 2 to 5 inclusive.
13. PAF or antibodies prepared using as antigen an antigenic PAF analogue of general formula (I) as defined according to claim 4 or claim 5.
14. A method for the preparation of PAF antibodies which method comprises:
introducing an antigen selected from:
 - (a) PAF adsorbed onto or non-covalently bound to a monofunctional or polyfunctional protein,

peptide, carbohydrate, lipid or a derivative thereof of molecular weight at least 2000 and capable of eliciting an antigenic response; and

- (b) the antigenic PAF analogues of general formula (I) as defined according to any one of claims 2 to 5 inclusive;
into an animal; and
isolating the antibodies produced in response to said antigen.

15. A method for the preparation of PAF antibodies which method comprises:

introducing an antigen selected from the antigenic PAF analogues of general formula (I) as defined according to claim 4 or claim 5 into an animal; and isolating the antibodies produced in response to said antigen.

16. PAF antibodies as defined according to claim 12 or claim 13 which have been labelled with a radioactive, enzymic or photometric label.

17. PAF antibodies as defined according to claim 12 or claim 13 which are polyclonal.

18. PAF antibodies as defined according to claim 12 or claim 13 which are monoclonal.

19. A method for the immunoassay of PAF in biological fluids which method comprises using a PAF antibody as defined according to any one of claims 12, 13 and 16 to 18 inclusive.

20. A method for the immunoassay of PAF in biological fluid wherein said biological fluid is diluted with a detergent before subjecting said biological fluid to immunoassay.
21. A method according to claim 20 wherein said detergent is a non-ionic detergent.
22. A method according to claim 20 or 21 wherein said detergent is selected from the group consisting of: polyalkylene glycols; alcohol, phenol and alkylphenol alkoxylates; castor oil alkoxylates; the partial esters derived from long chain fatty acids and hexitol anhydrides and their alkoxylates; long chain alcohol polyglycol ether acetals; alcohol sugar acetals; and the lecithins.
23. A kit for the immunoassay of PAF in biological fluid said kit comprising PAF antibodies as defined according to any one of claims 12, 13 and 16 to 18 inclusive.
24. Compounds of general formula (I) according to any one of claims 1 to 9 inclusive substantially as herein described with reference to Examples 1 to 4.
25. Method according to claim 10 for the preparation of compounds of general formula I substantially as herein described with reference to Examples 1 to 4.
26. PAF antibodies according to any one of claims 12, 13 and 16 to 18 inclusive substantially as herein described with reference to Example 6.

27. Method according to claim 14 or claim 15 for the preparation of PAF-antibodies substantially as herein described with reference to Example 6.
28. Method for the immunoassay of PAF in biological fluids according to any one of claims 19 to 22 inclusive substantially as herein described with reference to Example 6 or Example 7.

INTERNATIONAL SEARCH REPORT

International Application No PCT/AU 87/00084

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl. 4 C07F 9/10, G01N 33/92, C07K 15/12

II. FIELDS SEARCHED

Minimum Documentation Searched *

Classification System	Classification Symbols
IPC	C07F 9/10, G01N 33/92, G01N 33/16, C07K 15/12, C07G 7/00

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched *

AU : IPC as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT*

Category *	Citation of Document, ** with indication, where appropriate, of the relevant passages ***	Relevant to Claim No. *
X	US,A, 4370311 (ILEKIS) 25 January 1983 (25.01.83) See column 2 lines 11-18	(20-22)
A	Journal of Immunology Vol. 134 No.2 (1985) M. Odo et al, "Molecular Species of Platelet-Activating Factor Generated by Human Neutrophils challenged with Ionophore A23187" pages 1090-3	
A	US,A, 3708558 (KNY) 2 January 1973 (02.01.73)	
A	CA,A, 1169433 (GOVERNMENT OF THE UNITED STATES OF AMERICA) 19 June 1984 (19.06.84)	
A	Patent Abstracts of Japan, C-9, page 117, JP 55-28955 (TOYAMA KOGAKU KOGYO K.K.) 29 February 1980 (29.02.80)	
A	US,A, 4329302 (HANAHAN) 11 May 1982 (11.05.82)	
A	Chemical Abstracts, Volume 104, No.5 issued 1986, Lakin K. et al, "Activation of Rabbit Platelets induced by 1-O-alkyl-2-O-acetyl-sn-glycerophosphocholine" see page 388, abstract No. 32325s, Byull. Eksp. Biol. Med., 1985 100(10)410-12 (Russ).	

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubt on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"Z" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

18 June 1987 (18.06.87)

Date of Mailing of this International Search Report

(02.07.87) 2 JULY 1987

International Searching Authority
Australian Patent Office

Signature of Authorized Officer

J.G. HANSON

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. Claim numbers because they relate to subject matter not required to be searched by this Authority, namely:

2. Claim numbers because they relate to parts of the International application that do not comply with the prescribed requirements to such an extent that no meaningful International search can be carried out, specifically:

3. Claim numbers because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²

This International Searching Authority found multiple inventions in this International application as follows:

- (1) Claims 1 to 11, 12(b), 13, 14(b), 15 to 19, and 23 to 28
- (2) Claims 12(a) and 14(a)
- (3) Claims 20 to 22

1. As all required additional search fees were timely paid by the applicant, this International search report covers all searchable claims of the International application.

2. As only some of the required additional search fees were timely paid by the applicant, this International search report covers only those claims of the International application for which fees were paid, specifically claims:

3. No required additional search fees were timely paid by the applicant. Consequently, this International search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

The additional search fees were accompanied by applicant's protest.

No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON
INTERNATIONAL APPLICATION NO. PCT/AU 87/00084

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Members					
US	3708558	BE	763578	CH	542247	DE	2009341
		ES	388446	GB	1280788	IL	36299
		NL	7102495	YU	343/71	ZA	7101241
US	4329302	US	4504474	US	4551446		

END OF ANNEX